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Upregulation of cyclin D1, a key regulator of cell cycle, by elevated β-catenin through the disruption of Wnt signaling pathway is often seen in colon cancer and breast cancer. Cyclin D1 overexpression is also reported in about 25% of human prostate cancer where only about 5% mutation in β-catenin is found. Human AXIN2 and other components in the Wnt signaling pathway may also contribute to cyclin D1 elevation. To test this hypothesis, we checked 115 prostate cancer patients their cyclin D1 expression level in primary tumor cells using immunohistochemical staining by cyclin D1 antibody. Total number of 43 tumor samples were identified with strong and moderate cyclin D1 overexpression. Mutational analysis by DHPLC method revealed novel mutations in exon 1 of *AXIN2* gene (Ser50Pro and Ser81Pro) and exon 3 of *β-catenin* (Asp32Gly and Thr41Ala). Expression contructs of AXIN2 containing these mutations have been generated and functional roles of these mutant proteins in Wnt signaling pathway are being investigated.

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## Introduction

Accumulation of genetic changes affecting the expression and function of critical genes involved in cell proliferation and apoptosis is thought to drive malignant transformation and cancer progression. Such genetic alterations are poorly understood in prostate cancer. Analysis of such affected genes might confer deeper insight into these processes and might lead to better therapeutic strategies. The Wnt signaling pathway is a very conserved pathway and plays an important role in early development and tumorigenesis. Wnt regulates developmental and oncogenic processes through its downstream effector, β-catenin. Elevated β-catenin in the cytoplasm is translocated into the nucleus where it activates target genes such as *cyclin D1, c-myc*, etc., resulting in cancer. The upregulation of cyclin D1, a key regulator of cell cycle, by elevated β-catenin through the disruption of the Wnt signaling pathway is often seen in colon cancer and breast cancer. *Cyclin D1* overexpression is also reported in about 25% of human prostate cancer where only ~5% mutation in *β-catenin* is found. It suggests that other components in the Wnt signal pathway may also contribute to cyclin D1 overexpression. The human *AXIN2* gene cloned in our laboratory was mutated in about 30% of colorectal cancer with defective DNA mismatch repair (MMR). Our investigations show that this gene is involved in the Wnt signal pathway since mutant AXIN2 elevates nuclear β-catenin levels. Therefore the underlying hypothesis driving this project is that the mutant human *AXIN2* gene is involved in prostate carcinogenesis by upregulating cytosolic β-catenin that leads to cyclin D1 overexpression in the nucleus. Prostate tumors with elevated expression of cyclin D1 are more likely to harbor *AXIN2*, *AXIN1* and *β–catenin* mutations that may result in higher expression of β–catenin. The specific aims of this proposal are to: (1) characterize the expression of cyclin D1 in more than 100 collected prostate cancer samples; (2) screen for mutations in human *AXIN2*, *AXIN1* and *β-catenin* genes in samples with cyclin D1 overexpression; (3) construct mutant AXIN2 expression plasmids to elucidate its functional role in cell proliferation and apoptosis. We report here completion of characterization of prostate tumors with cyclin D1 overexpression using immunohistochemical staining method and identification of novel mutations of *AXIN2* and *β–catenin* in these tumors by DHPLC.

**Analysis of Human AXIN2 and Other Wnt Signal Pathway Genes in Human Prostate Cancer**
**(Award No. DAMD17-02-1-0092)**

Cyclin D1 is a member of a large family of cyclins. It forms complexes with cyclin-dependent kinases CDK4 and CDK6 to phosphorylate retinoblastoma protein allowing cells to proceed to S-phase (1-3). Deregulated overexpression of Cyclin D1 protein is frequently observed in breast, head and neck, colon, skin, and urinary bladder tumors (4-6). However, little is known concerning the expression status of cyclin D1 in primary prostate carcinomas. We collected 115 prostate cancer patients' paraffin-embedded tissue blocks and corresponding H&E slides from Mayo Clinic Tissue Registry. H&E slides were used to validate the presence of infiltrating carcinoma cells and score the pathological stage of these cells on the paraffin-embedded specimens as previously descrbed (7). For each patient, one tissue block was selected for the immunostaining study. A mouse monoclonal anti-human cyclin D1 antibody (Clone DCS-6, DAKO) was used for immunohistochemistry (8). The specificity of the antibody was verified by immunoblotting using prostate benign and carcinoma cell lines BPH-1, LnCap, DU145 and PC3. We optimized staining procedures concerning antibody dilutions and specific treatment of the tissue sections using paraffin-embedded specimens of breast carcinomas. The staining pattern of this antibody is predominantly nuclear. Cytoplasmic staining in the absence of nuclear staining is considered negative. We categorized 115 cases into three groups based on the cyclin D1 staining intensity. The first group displays the strongest nuclear staining when compared with neighboring normal cells (Figure 1a). The second group shows moderate intensity of nuclear cyclin D1 staining (Figure 1b). These two groups account roughly 37% of the cases (Table 1). The third group includes all cases that either have extremely weak or negative cyclin D1 staining (Figure 1c). Cases that have few tumor cells with positive staining are also considered negative. In majority of the cases (40 out of 43) with cyclin D1 positive staining, the pattern is extremely focal and represent only less than 10% of the carcinoma cells. The focal staining is usually observed in infiltrating carcinoma with clusters of very small glandular tubules. No apparent association is found between cyclin D1 staining and the Gleason grade of these 115 primary prostatic tumors ($p= 0.545$). We are currently examining 20 metastatic prostatic tumor samples and final data and analysis including these samples will be presented in the final report.

**Table 1. Characterization of immunohistochemical staining intensity for cyclin D1 in total 115 cases of primary prostatic carcinomas.**

| Cyclin D1 staining | Strong | Moderate | Negative |
|---|---|---|---|
| Prostate tumor | 2 (2%) | 41 (36%) | 72 (62%) |


**Figure 1. Immunohistochemical stained paraffin-embedded sections of primary prostatic carcinoma for cyclin D1. (a) One case shows high level of overexpression of cyclin D1 protein in prostate tumor cells compared with adjacent normal cells; (b) Moderate expression of cyclin D1 protein in another case; (c) weak or no expression of nuclear and cytoplamic cyclin D1 protein in prostatic tumor cells.**

We screened for mutations of *AXIN2* and exon 3 of *β-catenin* in isolated genomic DNA from 43 tissue samples that showed strong and moderate cyclin D1 staining stated in Table 1, using highly accurate and cost-effective DHPLC method. The primers and procedures used in the PCR amplification and DHPLC analysis are as described in the original proposal. A novel single nucleotide mutation T148C was found in the first exon of *AXIN2* in 2 patients and another one T241C, also in the first exon, was detected in 1 patient (Figure 2 (a) and 2 (b)). Both mutations cause amino acid change from serine to proline at positions 50 and 81, respectively. These two mutations occur in the APC binding domain of AXIN2 (9) and eliminate two phosphorylation sites that may have functional importance in Wnt signaling. The 12-bp deletion mutation in *AXIN2* (see the original proposal) were also detected in two patients (data not shown). Two different mutations were also found in the third exon of *β–catenin*, A95G and A121G, in two patients (Figure 2 (c) and 2 (d)). They switch amino acid from aspartic acid (32) to glycine and threonine (41) to alanine, respectively. They are present in the N-terminus that regulates the stability of β–catenin (10). These mutations in *AXIN2* and *β–catenin* do not co-exist in the same patient. Our current data indicates that *AXIN2* and *β–catenin* mutations are infrequent in primary prostate carcinomas (<8%).

We generated two different expression constructs of mutant AXIN2 with the 12-bp deletion, Ser50Pro and Ser81Pro in both pCMV and pTRE vectors. The pCMV expression construct can be used to transfect a wide spectrum of cell lines to constitutively express AXIN2 wild-type and mutant protein. The advantage of pTRE construct is that expression of AXIN2 is controllable by doxycycline, a chemical derivative of tetracycline, in Tet-On and Tet-Off cell lines (11). Using these two expression systems we observed that axin2 protein can be highly expressed in 293T, COS-7 and HeLa cells and both wild-type and mutant are localized in the cytoplasm. We are currently in the process of analyzing the function of the mutant AXIN2 in cell proliferation and apoptosis using these two expression systems by performing transient and stable transfections.

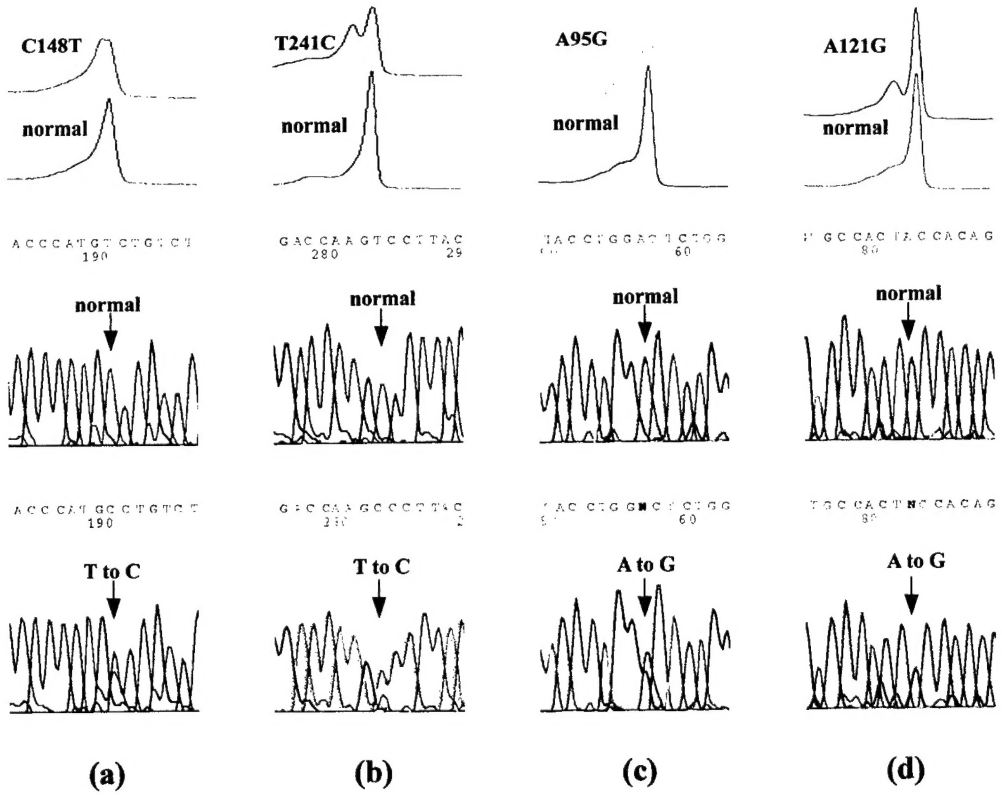


**Figure 2. DHPLC profiles and DNA sequences of exon 1 of *AXIN2* [(a) and (b)] and exon 3 of *β-catenin* [(c) and (d)] showing four newly detected single nucleotide change mutations. (a) T148C (Ser50Pro); (b) T241C (Ser81Pro); (c) A95G (Asp32Gly); (d) A121G (Thr41Ala).**

To summarize, we have completed Task 1, Task 3 and Most part of Task 2 as stated in the Statement of Work in the original proposal. The delay to accomplish Task 2 is due to the process of change of PI of this award in September in 2002 from previous PI Dr. Xiangyang Dong to me. We are still screening for *AXIN1* mutations (Task 2) in these selected primary prostate tumors. We will focus on Task4 and accelerate the remaining work in the second calendar year of this award.

Note: This report includes around 50% contribution of experimental work of Dr. Xiangyang Dong. Change of PI from Dr. Xiangyang Dong to Dr. Xianshu Wang for this award was completed in September, 2002.

## Key Research Accomlishments

1. Collection and preparation of 115 paraffin-embedded tumor tissue blocks from 115 prostate cancer patients for immunohistochemical staining purpose.
2. Determination of pathological stage of each and every paraffin-embedded tissue block.
3. Completion of immunostaining with mouse monoclonal antibody against human cyclin D1 (Clone DCS-6, DAKO) for all 115 paraffin-embedded tumor tissue block samples.
4. Fluorescence microscopic analysis of cyclin D1 staining results and identification of 43 tumor samples with strong and moderate overexpression of cyclin D1.
5. Mutational screening of *AXIN2* and *β–catenin* genes in the aforementioned prostate tumor samples with elevated cyclin D1 expression.
6. Identification of 4 novel missense mutations in exon 1 of *AXIN2* and exon 3 of *β–catenin.*
7. Generation of expression constructs of AXIN2 containing newly identified mutations Ser50Pro and Ser81Pro.
8. Transient expression of wild-type and mutant AXIN2 protein in 293T, COS-7 and HeLa cell lines.

## Reportable outcomes

## Conclusions

Publications on the status of cyclin D1 expression in primary prostate carcinomas are rare (12). The number of tumor samples and antibody used in cyclin D1 staining is of great importance in obtaining reliable data. We collected a large number of paraffin-embedded tissue blocks and selected a mouse monoclonal antibody (Clone DCS-6, DAKO) that has been demonstrated to work well in immunohistochemical staining (4-6). The immunostaining results showed that cyclin D1 overexpression occurred in about one-third of the prostate tumors we examined. The frequency is only half of the 60% rate observed in breast cancer. Even in tumors with increased expression of cyclin D1, positive staining of tumor cells is confined and focal, consistent with the heterogeneous nature of prostate cancer. The mutation rate of *AXIN2* and *β–catenin* is also lower than that in colon cancer indicating that genetic alterations contributing to the majority of non-Mendelian inheritance of prostate cancer are very likely the common low penetrant alleles. Genetic components in pathways other than Wnt signaling, such as DNA damage and repair signaling pathway, might also play critical roles in prostatic carcinogenesis. Our future work will focus on validating the significance of these mutations by screening a larger group of prostate cancer patients and normal control population. In the meantime, we are concentrating on finding the functional implications of these mutations in cell growth, proliferation and apoptosis.

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## Appendices

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